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STUDIES ON THE STRUCTURE AND CONFORMATION OF YEAST MITOCHONDRIAL ATPase USING AUROVERTIN AND METHANOL AS PROBES

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Summary

1. The isolation of the mitochondrial ATPase F_1 and its β-subunit from commercial baker's yeast (*Saccharomyces cerevisiae*) is described.

2. The molecular weight determined by ultracentrifugation is 340 000 ± 30 000. Gel chromatography indicates a molecular weight of 300 000 ± 20 000.

3. Fluorimetric titration of the isolated enzyme with aurovertin reveals two binding sites per molecule. The isolated β-subunit binds aurovertin in a 1 : 1 stoicheiometry. It is concluded that the ATPase molecule contains two aurovertin-binding β-subunits.

4. The stabilizing agent methanol influences both the measured K_d and the concentration of binding sites for aurovertin. These results fit a model in which both F_1 and aurovertin are distributed between aqueous and methanol phases.

5. The effect of methanol on the ATPase activity can be described in terms of the model proposed by Recktenwald and Hess (Recktenwald, D. and Hess, B. (1977) FEBS Lett. 76, 25—28). It is proposed that methanol enhances the affinity of the regulatory site for ATP, but at higher concentrations prevents the interaction between the regulatory and catalytic sites.

6. Since HSO_3^-, a typical effector of the assumed regulatory site of F_1, has no effect on the binding of aurovertin, it is concluded that the binding site of aurovertin is not correlated with the regulatory site.

7. The inhibition of ATPase activity by aurovertin is slowly ($t_{1/2}$ = 70 s) induced during turnover conditions.

8. From the effect of methanol on the inhibition of ATPase activity by aurovertin it is concluded that under turnover conditions the conformation is such that the aurovertin-binding sites have a 6-fold higher affinity for methanol than under resting conditions.

Introduction

The antibiotic aurovertin inhibits ATP synthase from various sources [1—6] and its binding to the enzyme is mostly [2,7,8], but not in chloroplasts or *Escherichia coli* [9], accompanied by a large increase of its fluorescence. The sensitivity of this fluorescence to conformational changes in the enzyme has made it a widely used tool in studies on the properties of this complex enzyme [2,7,10—12]. It has been shown that the isolated beef-heart enzyme binds 2 mol aurovertin per mol [13] and that both sites are located on the β-subunit [14]. The binding of aurovertin is then a simple tool for measuring the concentration of F_1 in a mitochondrial preparation [15].

Since for beef-heart F_1 the aurovertin-binding studies suggest the presence of two β-subunits, whilst for bacteria the data obtained from radioactive labelling favour the postulate of three α- and three β-subunits per F_1 [16], we undertook a study of yeast F_1: with this organism both methods can be used. Douglas et al. [17,18] showed that in yeast F_1, just as in beef-heart F_1, the β-subunit is responsible for the binding of aurovertin. Thus, with yeast F_1 it is possible to find out whether the number of aurovertin-binding sites in native and LiCl-dissociated F_1 is identical with the total number of β-subunits determined by radioactive labelling experiments. In this paper we show that the β-subunit may be isolated in such a way that it binds one molecule of aurovertin and that under the same conditions both intact and dissociated F_1 bind 2 mol aurovertin per mol F_1, just as with heart F_1. A preliminary report has been published [19]. Studies on radioactive labelling (unpublished observations) show the presence of three β-subunits and three α-subunits per mol F_1. Possible reasons for this difference between the number of aurovertin-binding sites and the number of β-subunits are discussed in the present paper.

Since methanol is often used to stabilize isolated yeast F_1 [20], it was necessary to study its effect on the enzyme, especially on the binding of aurovertin and on the ATPase activity. Particular attention was given to a possible relationship between the aurovertin-binding site and the regulatory [21] and catalytic sites on F_1.

Materials and Methods

Preparation of mitochondria

5 kg yeast (purchased from Bruggeman, Gent, Belgium) were suspended in 10 l medium A (500 mM sucrose/100 mM Tris-H_2SO_4 buffer, 0.2% bovine serum albumin, 2 mM EDTA, 1 mM ATP and 0.1 mM phenylmethylsulphonylfluoride, pH 7.5). The cells were broken with a Dyno Mill (Willy A. Bachhoven, type KDL). This system has been described by Deters et al. [22], with the difference that an extra neoprene ring has to be inserted to avoid leakage. The cooling system used was a Kotterman KT 50 S cryostat, with methanol as cooling liquid. The 600 ml glass breakage chamber was used, filled with glass beads of 0.52—0.69 mm diameter. The speed of rotation of the stirring discs was 3000 rev./min (15 m/s) and the flow rate of the yeast suspension was 6 l/h. Under these conditions an optimal yield of heavy mitochondria was obtained. After passing through the Dyno Mill, the suspension was cooled to 4°C and

centrifuged at 2500 × *g* for 30 min. From the supernatant the mitochondria were collected by centrifugation for 30 min at 16 000 × *g* and suspended in medium A. After washing, the mitochondria were stored at −20°C as a concentrated suspension (60 mg/ml). The yield was 10 g mitochondrial protein per kg pressed yeast.

Isolation of yeast mitochondrial ATPase

After thawing, the mitochondria were washed once more with medium A, and the suspension (40 mg/ml) was brought to pH 6.4 and sonicated for 10 s at 0°C with a Branson Sonifier, type B 12, at the lowest energy output. After readjusting the pH to 7.5, the particles were collected by centrifugation for 30 min at 150 000 × *g* and suspended in a medium containing 600 mM sorbitol, 10 mM Tris-H_2SO_4 buffer, 2 mM dithiothreitol, 1 mM EDTA, 4 mM ATP and 0.1 mM phenylmethylsulfonylfluoride brought to pH 7.5. The presence of a high concentration of sorbitol was found essential to protect F_1 against the next step, the extraction with 33% chloroform, which, except for this addition of sorbitol, was carried out as described by Takeshige et al. [23]. The temperature was carefully kept at 15—17°C. After a low-speed centrifugation to separate the chloroform from the water phase, the latter was stirred for about 2 h under a stream of N_2 to remove chloroform. Insoluble material was then removed by centrifugation at 150 000 × *g* for 30 min.

The resultant, slightly yellowish clear extract was introduced onto a DEAE-cellulose column (3.5 × 5 cm), equilibrated with medium B (10 mM Tris-H_2SO_4 buffer, 2 mM dithiothreitol, 2 mM ATP, 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride brought to pH 7.5). The column was then washed with medium B, containing 10% methanol, followed by medium B, containing 10% methanol and 20 mM K_2SO_4. The ATPase was eluted when the K_2SO_4 concentration was increased to 80 mM. Fractions with enzyme activity were pooled and 10% glycerol was added for stabilization during the next step of concentration via ultrafiltration at 4°C (Amicon filtration set, PM 30 filter). The concentrated solution (about 1 ml) was brought on a ACA-34 column (1.6 × 120 cm), equilibrated with a solution containing 50 mM Tris-H_2SO_4 buffer, 0.5 mM EDTA, 1 mM ATP, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 10% methanol and 10% glycerol brought to pH 7.5, and elution was carried out with the same solution. The fractions containing ATPase activity were pooled and concentrated. For further purification the last step was repeated. The final preparation was stored in liquid nitrogen. The yield of the various steps and the specific activity at each state of the isolation are given in Table I.

Isolation of the β-subunit of F_1

The subunit with molecular weight of 50 000 (β) was isolated by a procedure similar to that described by Verschoor et al. [14] for the β-subunit of beef-heart F_1. The yeast F_1 was precipitated from 85% satd. $(NH_4)_2SO_4$ by centrifugation at 60 000 × *g* and 0°C for 30 min, and dissolved to 12 mg/ml in 250 mM sucrose, 10 mM Tris-H_2SO_4 buffer, 4 mM ATP, 2 mM EDTA and 850 mM LiCl brought to pH 8.0. After one night at 16°C (4 h gives the same result), the mixture was applied on a ACA-44 column (1.4 × 105 cm), equilibrated with

TABLE I

ISOLATION OF ATPase F_1 FROM COMMERCIAL BAKER'S YEAST, *SACCHAROMYCES CEREVISIAE*

Treatment	Preparation	Specific activity (μmol $P_i \cdot mg^{-1} \cdot min^{-1}$)	Yield of activity (%)	Protein (mg)
Disruption yeast cells	Mitochondria	1.3	100	5150
Sonification	Submitochondrial particles	1.5	74	3200
$CHCl_3$ extraction	Extract	10.8	70	430
DEAE-chromatography and concentration	Concentrate	95.5	42	30
Gel filtration ACA 34	1X-ACA	138	36	17
Gel filtration ACA 34	2X-ACA	154	28	12

the same medium. Elution was performed with the same medium, and all fractions showing absorbance at 288 nm were tested for enhancement of aurovertin fluorescence. The fractions showing enhancement of aurovertin fluorescence were pooled and found to contain the β-subunit in pure form (Fig. 1C and 1D).

ATPase activity

ATPase activity was measured at 30°C spectrophotometrically, using the regenerating system coupled to the oxidation of NADH. The reaction mixture contained 83 mM sucrose, 16.7 mM Tris-HCl buffer, 0.1 mM free Mg^{2+}, different concentrations of MgATP, 18 units (μmol/min) pyruvate kinase, 36 units lactate dehydrogenase, 1.5 mM phospho*enol*pyruvate and 0.27 mM NADH in a total volume of 3 ml. The final pH was 8.0 and the reaction was started with the addition of the preparation to be tested. In routine measurements of ATPase activity the concentration of MgATP was 5 mM. The activity is expressed in μmol $P_i \cdot min^{-1} \cdot mg^{-1}$, except for purified F_1. In the latter case the activity is expressed as turnover number (s^{-1}), assuming a molecular weight of 340 000.

Protein

Protein was determined according to Lowry et al. [24] using bovine serum albumin Grade V as a standard (A = 0.667 ml $\cdot mg^{-1} \cdot cm^{-1}$ at 279.5 nm).

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Laemli [25], in a Biorad slab-gel apparatus (model 221). The concentration of acrylamide was 12% (w/v) for the running gel and 3% for the stacking gel. Staining with 0.12% Coomassie blue and destaining were carried out in 10% acetic acid and 20% (v/v) methanol. To avoid breakdown of polypeptides by contaminating proteases in the presence of 0.1% SDS, the samples were incubated shortly before application on the gel. Gel electrophoresis in the presence of both dodecyl sulphate and 8 M urea was performed as described by Swank and Munkres [26], using a 10 : 1 ratio of acrylamide to bisacrylamide, in rods of 190 × 5 mm.

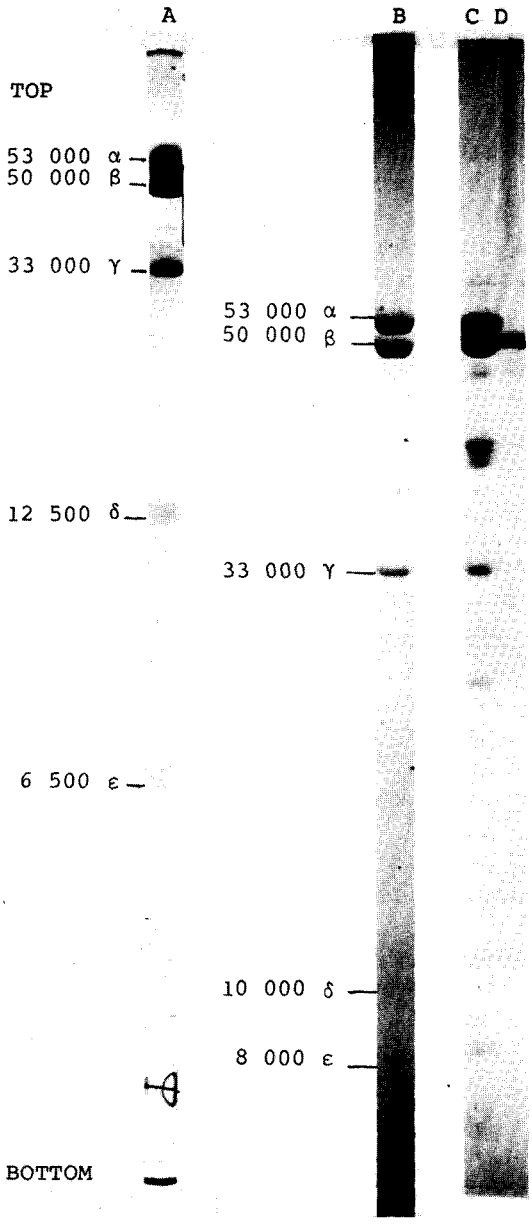

Fig. 1. Subunit pattern of yeast ATPase F_1. The position of the subunits and their molecular weights are indicated by arrows. A. Urea-SDS-polyacrylamide gel (8 M urea, 8% polyacrylamide), 40 μg of ATPase (spec. act. 150 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$). B. Slab gel, 12% polyacrylamide, 0.1% SDS, 20 μg of the same preparation of ATPase as in A. C. Slab gel, 20 μg of ATPase (spec. act. 185 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$). This preparation was used to isolate the β-subunit. D. Slab gel, 4 μg β-subunit.

Fluorescence measurements

Fluorescence measurements were carried out at 17°C in an Eppendorf fluorimeter. The sample was stirred continuously. Aurovertin fluorescence was measured with excitation at 366 nm (filter 313 + 366 nm) and emission between 430 and 3000 nm.

DEAE-cellulose was obtained from Serva (Servacel, type 23 SH), acrylamide and bisacrylamide from BioRad, California (U.S.A.) and ultragel ACA 44 and ACA 34 from LKB, Uppsala (Sweden). Pyruvate kinase (in 50% glycerol) and lactate dehydrogenase (from pig heart, in $(NH_4)_2SO_4$) were obtained from Boehringer, Mannheim and the protease inhibitors phenylmethylsulphonylfluoride, *p*-aminobenzamidine and ϵ-aminocaproic acid from Sigma (U.S.A.). Aurovertin D was isolated in this laboratory by R.M. Bertina following the method described in Ref. 10 and its concentration was determined spectrophotometrically, using an absorbance coefficient of 29 $mM^{-1} \cdot cm^{-1}$ at 367.5 nm [13].

Results

Composition and molecular weight

Yeast F_1 as isolated contained 1.6 mol tightly bound ATP and 1.4 mol tightly bound ADP per mol. Gel filtration in the absence of ATP removed 90% of the bound ADP, which is to be expected if the dissociation constant of the ADP is the same as that found for beef-heart F_1 by Wielders [27].

The molecular weight of the isolated enzyme was determined both by molecular sieving on ACA-34 and sedimentation. The yeast F_1 migrated slightly more slowly than the beef-heart F_1 on ACA-34, both in the presence and absence of 2 mM ATP in the eluting buffer. Assuming 343 000 as the molecular weight of beef-heart F_1, and the values given by the delivering company for the standard proteins, the molecular weight of yeast F_1 was found to be 300 000 ± 30 000. This is considerably smaller than that reported by Takashige et al. [23], namely 400 000, although the R_F values of their preparation and ours are very similar. The difference in the calculated molecular weight is due to a difference in the calibration curves.

In the analytical ultracentrifuge a sedimentation coefficient of 10.5 ± 0.5 S was found. Using the value given by Takeshige et al. [23] for D ($2.92 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$), v (0.738 $cm^3 \cdot g^{-1}$) and ρ (1.00 $g \cdot cm^{-1}$), a value of 340 000 ± 34 000 for the molecular weight may be calculated.

For the calculations of the turnover number and the number of aurovertin-binding sites, a molecular weight of 340 000 is assumed.

Five stainable subunits can be distinguished on SDS-polyacrylamide slab-gel and urea-SDS-polyacrylamide rod-gel electrophoresis. In both gel systems the α-, β- and γ-subunits have the same apparent molecular weights as the beef-heart enzyme (53 000, 50 000 and 33 000, respectively) but the δ-subunit is smaller and the ϵ-subunit is larger than in beef heart. The apparent molecular weights are 12 500 and 6500, respectively, on urea-SDS-polyacrylamide gels and 10 000 and 8000 on SDS-polyacrylamide gels (see Fig. 1A and B). The bands that are just visible between the β- and γ-subunits certainly represent impurities since they become less intense after each passage through ACA-34, without decrease in specific activity. These bands are also present when F_1 is isolated in the presence of phenylmethylsulphonylfluoride, *p*-aminobenzamidine and ϵ-aminocaproic acid, in concentrations equal to those used by Ryrie [20].

Binding of aurovertin to F_1 and the β-subunit

Addition of aurovertin to a solution of F_1 causes a biphasic increase of the fluorescence (see below, Fig. 7A) similar to that found with the beef-heart enzyme [13] but with a slightly faster second phase. The final enhancement of the fluorescence obtained with different amounts of aurovertin was used to determine the concentration of binding sites. Extrapolation to infinite protein concentration of the fluorescence obtained with the same concentration of aurovertin and varying concentrations of protein was used to determine the fluorescence of bound aurovertin. This is 60 times that of aurovertin in solution. The Scatchard plots shown in Fig. 2 indicate the presence of 2.0 mol binding sites per mol F_1 assuming a molecular weight of 340 000, with a K_d of 0.19 μM. The addition of MgATP or ATP caused a decline of the fluorescence yield of aurovertin by 15% without effect on the concentration of binding sites or the K_d (not shown).

Dissociation of F_1 with LiCl results in a disappearance of the fluorescence enhancement unless ATP is present during the dissociation. After dissociation in the presence of ATP, 2 mol aurovertin can still be bound per mol F_1 (Fig. 3).

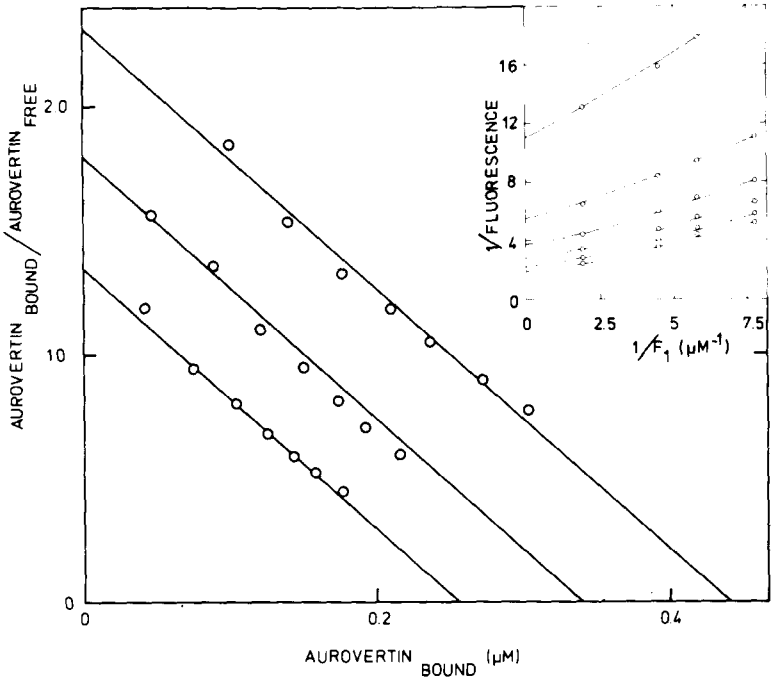

Fig. 2. Titration of yeast F_1 with aurovertin. Aurovertin (58.0 μM) was added stepwise to 0.14, 0.18, 0.23 μM F_1 (turnover number 0.94 ms^{-1}), respectively, in 1.5 ml medium containing 10 mM Tris-H_2SO_4 buffer, 2 mM EDTA, pH 7.5 at 17°C and 0.3, 0.4 and 0.5% methanol, respectively. The fluorescence was measured when a constant level was reached. The results of the titration are plotted as a Scatchard plot. The value for the fluorescence of bound aurovertin is derived from the plot shown in the inset and that for the fluorescence of the free aurovertin was determined separately. The enhancement of the fluorescene was 59.6 times. The drawn lines are calculated assuming that 1 mol of F_1 contains 2.0 mol binding sites for aurovertin and that the K_d is 0.19 μM. The inset shows the plot of the reciprocal of the amount of protein vs. the reciprocal of the fluorescence measured. Extrapolation to infinite protein gives the value for the fluorescence of bound aurovertin, needed for the calculations of the Scatchard plots. The drawn lines are calculated on the basis of the values for K_d and n, derived from the Scatchard plots. The concentrations of aurovertin are from top to bottom 0.155, 0.232, 0.309, 0.386 and 0.464 μM.

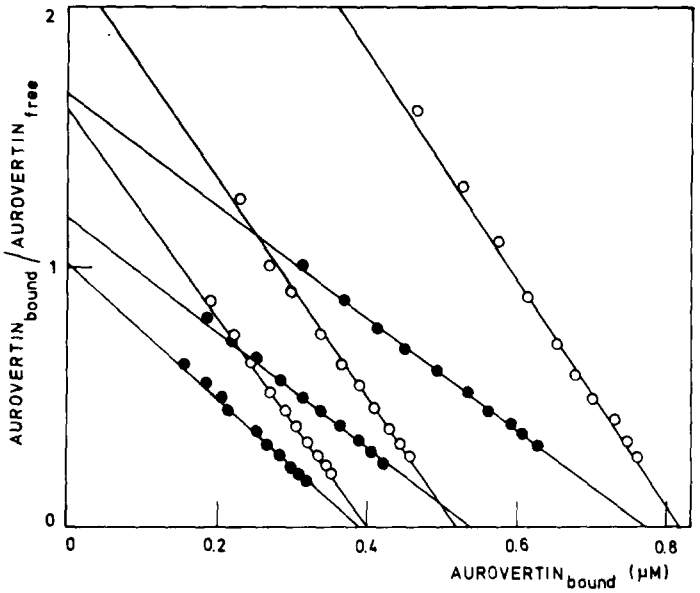

Fig. 3. Titration of yeast F_1 with aurovertin, before and after incubation in the presence of 0.85 M LiCl. Aurovertin (78.3 μM) was added stepwise to 0.20, 0.26 and 0.40 μM F_1 (turnover number 0.77 ms^{-1}) in 1.5 ml medium containing 5 mM Tris-H_2SO_4 buffer, 2 mM EDTA, 4 mM ATP, pH 8.25 before (○———○) and after (●———●) incubation with 0.85 M LiCl for 3 h at 17°C, and the fluorescence measured when a constant level was reached at 17°C. The results of the titration are plotted as a Scatchard plot. The enhancement of the fluorescence was 59 and 19 times, respectively. The two sets of plots yield 2.0 mol binding sites for aurovertin per mol yeast F_1. K_d = 0.25, 0.24 and 0.23 μM, respectively, in the absence of LiCl and 0.38, 0.45 and 0.46 μM, respectively, after incubation in the presence of 0.85 M LiCl.

After chromatography of F_1 dissociated in the presence of ATP, only those fractions containing the β-subunit of F_1 show enhanced fluorescence of aurovertin. As is the case with the β-subunit of beef-heart F_1 [14], the fluorescence increase is now monophasic, and the enhancement is only about 20-fold. Scatchard plots indicate the presence of 0.9 binding site per polypeptide of 50 000 molecular weight (Fig. 4), with a K_d value of 0.3 μM (cf. 0.19 μM for the β-subunit of heart F_1 [14]). Either the binding of aurovertin to the β-subunit or the fluorescence enhancement upon binding appears to be very sensitive to the state of the polypeptide, since upon removal of ATP from the medium the fluorescence enhancement disappears irreversibly.

The effect of methanol and anions

Since methanol appeared to have a stabilizing effect on F_1, its effects on the binding of aurovertin and the ATPase activity were studied. In the presence of 10% methanol the concentration of detectable binding sites is drastically lowered. This effect is reversible. Fig. 5 shows Scatchard plots for the binding of aurovertin obtained in the presence of various amounts of methanol. The number of binding sites detectable is decreased and the value of the dissociation constant increased with increasing methanol concentrations. The fluorescence of bound aurovertin, however, was not influenced by the presence of methanol.

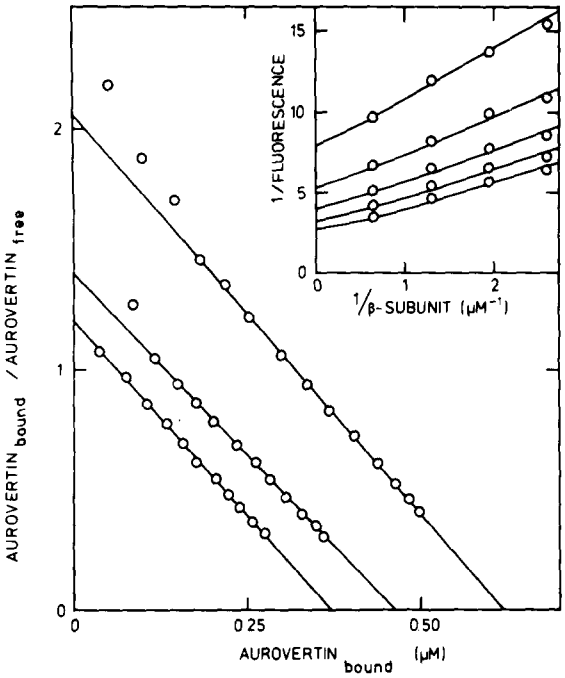

Fig. 4. Titration of the β-subunit of yeast F_1 with aurovertin. Aurovertin (58.0 μM) was added stepwise to 0.39, 0.51 and 0.77 μM β-subunit (left to right) in 1.5 ml medium containing 250 mM sucrose, 10 mM Tris-H_2SO_4 buffer, 2 mM EDTA, 4 mM ATP, 850 mM LiCl, pH 8.0 at 17°C, and the fluorescence measured when a constant level was reached. The results of the titration are plotted as a Scatchard plot. The value for the fluorescence of bound aurovertin, derived from the plot shown in the inset, was 81.0 units/μM. The enhancement of the fluorescence is 19.0 times. The three plots yield 0.96, 0.91 and 0.81 mol binding sites for aurovertin per mol β-subunit. K_d = 0.30, 0.33 and 0.30 μM, respectively. The inset shows the plot of the reciprocal of the concentration of protein vs. the reciprocal of the fluorescence measured. Extrapolation to infinite protein concentration gives the value for the fluorescence of the bound aurovertin (81.0/μM), needed for the calculations of the Scatchard plots. The drawn lines are calculated on the basis of the value of 0.3 μM for the K_d and of 0.9 mol binding sites for aurovertin per mol β-subunit. The concentrations of aurovertin are from top to bottom 0.077, 0.155, 0.232, 0.309 and 0.386 μM.

The increase of the K_d with increasing methanol concentration can be ascribed to a higher affinity of aurovertin for a water-methanol medium than for a water medium. Mathematically this higher solubility of aurovertin in a methanol-containing medium can be treated as a distribution of aurovertin over an aqueous phase and a methanol phase, where only the aurovertin in the aqueous phase is involved in the binding. Such a description of the effect of methanol is analogous to the description of aspecific binding [28]:

$$[\text{aurovertin}]_{free} = [\text{aurovertin}]_{H_2O} + [\text{aurovertin}]_{methanol} = [\text{aurovertin}]_{H_2O} \cdot (1 + C_1[\text{methanol}]),$$

where C_1 is the distribution of aurovertin between methanol and water at 1% methanol, and [methanol] is expressed as percentage (v/v).

$$K_d(\text{measured}) = [\text{aurovertin}]_{free} \cdot [\text{free sites}]/[\text{bound sites}] = [\text{aurovertin}]_{H_2O} \cdot (1 + C_1[\text{methanol}]) \cdot [\text{free sites}]/[\text{bound sites}] = K_d(H_2O) \cdot (1 + C_1[\text{methanol}]).$$

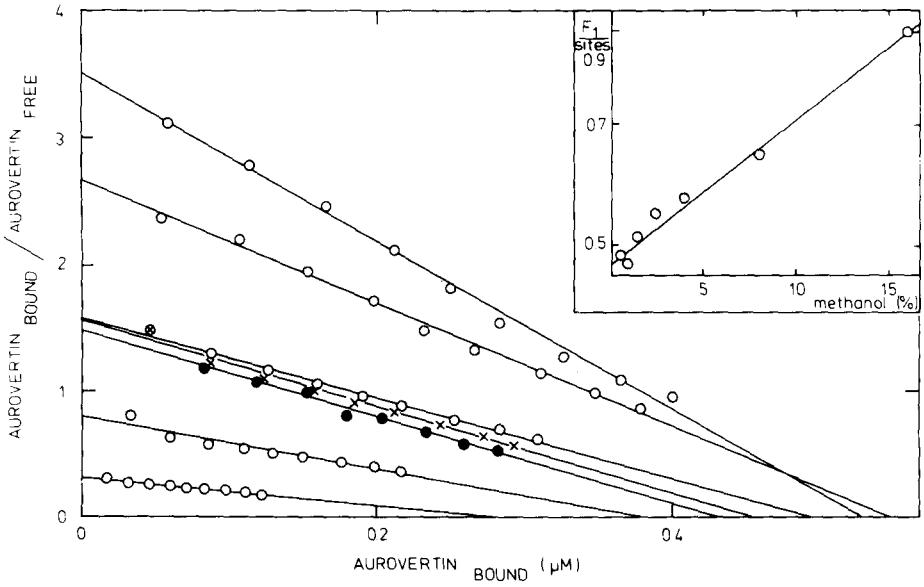

Fig. 5. Effect of methanol on binding of aurovertin to ATPase. Aurovertin (58.5 μM) was added stepwise to 0.25 μM F_1 (turnover number 0.94 ms^{-1}) in the presence of 0.6, 1.0, 1.5, 2.5, 4.0, 8.0 and 16.0% (v/v) methanol (curves from top to bottom) in 1.5 ml medium containing 10 mM Tris-H_2SO_4 buffer, 2 mM EDTA, pH 7.5 at 17°C. The results of the titration are plotted as Scatchard plots. The fluorescence enhancement is 60 times. The drawn lines are calculated according to the linear regression procedure. One mol of F_1 can bind 2.07, 2.15, 1.94, 1.78, 1.70, 1.49 and 1.11 mol aurovertin, with K_d values of 0.15, 0.20, 0.31, 0.29, 0.29, 0.48 and 0.93 μM, respectively. In the inset is plotted the reciprocal of the apparent number of binding sites for aurovertin vs. the concentration of methanol present in the medium. The drawn line is calculated according to the linear-regression procedure.

Plotting K_d(measured) against [methanol], the intersection with the ordinate gives $K_d(H_2O)$ and the slope equals $K_d(H_2O) \cdot C_1$. The experiment of Fig. 5 gives $K_d(H_2O) = 0.16$ μM and $C_1 = 0.3$.

The decrease in the concentration of binding sites may be due to a transition of the binding site to a different conformation, not able to bind aurovertin. Mathematically this can be treated as a binding of methanol to this site or to distribution of the site over an aqueous and a methanol phase:

$$[\text{sites}]_{\text{total}} = [\text{sites}]_{H_2O} \cdot (1 + C_2[\text{methanol}])$$

where C_2 equals the distribution of the sites over methanol and water at 1% methanol. Assuming that F_1 in H_2O contains n sites for aurovertin per molecule and that the sites in methanol are unable to binding aurovertin

$$n[F_1]_{\text{total}} = [\text{sites}]_{\text{total}} = [\text{sites}]_{H_2O} \cdot (1 + C_2[\text{methanol}])$$

$$\frac{[F_1]_{\text{total}}}{[\text{sites}]_{H_2O}} = (1 + C_2[\text{methanol}])/n \,.$$

Since the measured concentration of sites equals $[\text{sites}]_{H_2O}$, plotting $[F_1]_{\text{total}}/[\text{sites}]_{H_2O}$ against [methanol] yields a straight line with intersection with the ordinate equal to $1/n$ and a slope of C_2/n. The experiment of Fig. 5, plotted in this way in Fig. 5, inset, yields $n = 2.1$ and $C_2/n = 0.028$, so that $C_2 = 0.053$.

This means that in the absence of methanol F_1 contains two sites for aurovertin, but this number is lowered to one at about 18% methanol. The alternative explanation, namely that the aurovertin-binding site in the methanol phase binds aurovertin, but the bound aurovertin is non-fluorescent, is excluded by the finding that the fluorescence of bound aurovertin, i.e. the fluorescence in the presence of infinite protein, is not influenced by methanol.

The effect of methanol on the ATPase activity (measured with 5 mM Mg-ATP) is not simply related to the concentration of methanol. At low concentrations the activity is inhibited (maximal effect at 3% methanol) whereas at higher concentrations, up to 20%, it is stimulated. These effects of methanol are not caused by an effect on the regenerating system. Lineweaver-Burk plots (Fig. 6) show that methanol has no effect on the maximum velocity. With 10% methanol (Fig. 6B) the high K_m is absent, whereas with 3% methanol (Fig. 6A) the change from low to high K_m takes place at a concentration of ATP lower than that in the absence of methanol. The effect of 10% methanol is similar to that of 10 mM sulphite and can be explained according to the model proposed by Recktenwald and Hess [21], in which it is assumed that ATP can bind to a regulatory site of F_1 resulting in an increase in the K_m for ATP without any

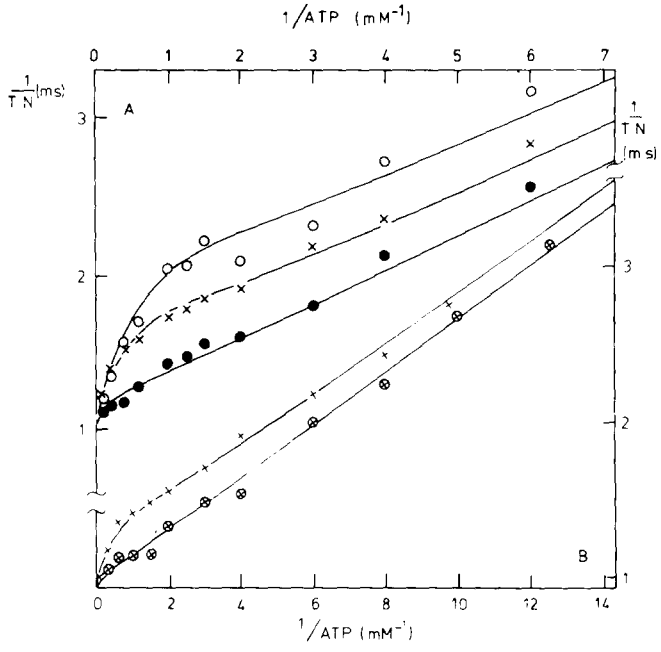

Fig. 6. A. Effect of methanol on ATPase activity at different ATP concentrations. Above 0.5 mM ATP, 0.15 nM ATPase was used; below that concentration 0.30 nM. T.N. turnover number. X———X, no further addition; ○———○, +3% (v/v) methanol; ●———●, +10 mM H_2SO_3 (9.3 mM SO_3^{2-} at pH 8). The drawn lines are calculated assuming $K_m(I) = 0.22$ mM (cf. 0.042 calculated in Ref. 20), $K_m(II) = 1.6$ mM and $V = 0.85$ ms^{-1}. For the K_d of the regulatory site for ATP a value of 1 mM is assumed when no addition is made, of 0.5 mM when 3% methanol is present and of 7 mM when 9.3 mM SO_3^{2-} is present. B. The same preparation as in Fig. 6A is used. X———X, no further additions; ⊗———⊗, 10% (v/v) methanol. The drawn lines are calculated assuming $K_m(I) = 0.18$ mM, $K_m(II) = 1.0$ mM and $V = 0.94$ ms^{-1}. For the K_d of the regulatory site a value of 1 mM is assumed in the absence of 10% methanol, of 10 mM in its presence.

effect on V. The relative concentration of F_1 with a high and a low K_m value is therefore determined by the concentration of ATP. The total ATPase activity can be calculated by summing up the activity of the two forms of F_1 present. The Lineweaver-Burk plots shown in Fig. 6 may be simulated by assuming certain values for V, K_d (of the regulatory site) and the two K_m values. According to this model, low concentrations of methanol increase the affinity of ATP for the regulatory site, but at higher concentrations this affinity decreases again, or, more likely, the binding of ATP no longer affects the affinity of the catalytic site for ATP. In the presence of sulphite, methanol has no effect (not shown), indicating that methanol and sulphite affect the same regulatory site. The effect of 10% methanol on the ATPase activity is temperature-dependent, at variance with the results of Gomez et al. [29]. Also the non-linearity of the Lineweaver-Burk plots in the absence of methanol does not change between 30 and 5°C, as is the case with the concentration of aurovertin-binding sites. Since sulphite was found to have no effect on the fluorimetric titrations with aurovertin, whether ATP was present or not, a correlation between the conformation of the regulatory site and the binding site for aurovertin is unlikely. The lowering by ATP of the fluorescence of bound aurovertin probably reflects a relation between the catalytic site and the aurovertin-binding site.

Effect of methanol on the inhibition of F_1 by aurovertin

Fig. 7 shows that the inhibition by aurovertin of the ATPase activity is a slowly developing phenomenon during turnover of the enzyme, with a half time of about 70 s. Pre-incubation of F_1 with aurovertin does not result in inhibition of the initial rate of ATP hydrolysis (not shown). The simplest model for binding of aurovertin and inhibition is expressed by the equations

$$F_1 + A \rightleftharpoons F_1 \cdot A \rightleftharpoons F_1^* \cdot A$$

where F_1^* is an inhibited form of F_1. It can be calculated from the K_d for aurovertin that, under the conditions of the experiment of Fig. 7, in the absence of turnover the ratio $[F_1]/[F_1 \cdot \text{aurovertin}]$ would be 1/4. Since the final inhibition equals 95%, $F_1 : F_1 \cdot A : F_1^* \cdot A = 1 : 4 : 95$.

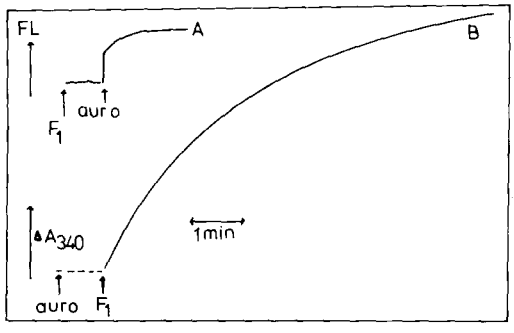

Fig. 7. Time course of the fluorescence increase and inhibition of ATPase activity after addition of aurovertin to F_1. A. Fluorescence enhancement upon addition of 0.080 μM aurovertin to 0.22 μM ATPase in a medium containing 10 mM Tris-H_2SO_4 buffer, 2 mM EDTA, pH 7.5, at 17°C. B. ATP hydrolysed by 0.22 nM ATPase in the presence of 0.78 μM aurovertin.

Methanol largely prevents inhibition by aurovertin (Fig. 8). This effect of methanol can be explained only partially by its effect on the binding of aurovertin in the resting state (increase of apparent K_d and decrease of aurovertin-binding sites). Using the same formalism as before, we may derive the following equations:

In the presence of methanol

$$\frac{[F_1 + F_1 \cdot A + F_1^* \cdot A]_{H_2O} + [F_1]_{methanol}}{[F_1^* \cdot A]_{H_2O}} = 1.053(1 + C_3)[\text{methanol}]$$

$$= 100/\% \text{ inhibition}$$

In the absence of methanol

$$[F_1 + F_1 \cdot A + F_1^* \cdot A]/F_1^* \cdot A = 1/0.95 = 1.053$$

where C_3 is the distribution coefficient of total F_1 under turnover conditions between the aqueous and the methanol phases. In the plot of 100/% inhibition against % methanol (Fig. 8), the intersection with the ordinate gives 100/% inhibition in the absence of methanol (1.053) and the slope gives $1.053\ C_3 = 0.34$, from which it follows that C_3 equals 0.32, which is six times higher than C_2 (0.053). This means that the sensitivity of the aurovertin-binding sites for methanol is increased drastically under turnover conditions.

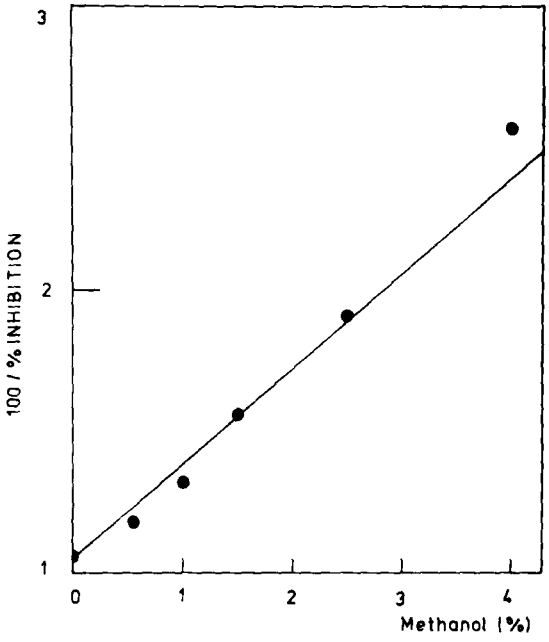

Fig. 8. Effect of methanol on inhibition by aurovertin on ATPase activity. The ATPase activity with 0.85 nM F_1 was measured, with and without 0.81 μM aurovertin, and with methanol as indicated. The activity in the absence of aurovertin but in the presence of methanol was set at 100%.

Discussion

The aurovertin-fluorescence titrations indicate the presence of two binding sites for aurovertin per molecule of F_1. It should be mentioned that for the calculation of the binding parameters from the titration data, it is very critical that the fluorescence of the bound aurovertin is calculated correctly. The procedure we adopted was first to obtain an approximate value by linear extrapolation of the plots fluorescence^{-1} against protein^{-1} to protein^{-1} = 0 and to use this value for the calculation of the Scatchard plots. From these plots preliminary values for K_d and n were derived. Finally both the Scatchard plots and the plots fluorescence^{-1} against protein^{-1} were simulated with slightly different values of n, K_d and fluorescence yield until the best fit with the experimental points was obtained. This simulation is needed since the plot fluorescence^{-1} vs. protein^{-1} is not a straight line for a three-component equilibrium. At variance with the results reported for beef-heart F_1 [13], the titrations with yeast F_1 show homogeneous binding of aurovertin, described with linear Scatchard plots. At the moment we have no satisfactory explanation for this difference.

From the experimeht shown in Fig. 3 it is clear that dissociation of F_1 in the presence of LiCl (and ATP) does not alter the concentration of binding sites for aurovertin, although the fluorescence of bound aurovertin and the binding constant change to values characteristic for the isolated β-subunit. Since the isolated β-subunit binds aurovertin with a stoicheiometry close to 1 : 1, the presence of two aurovertin-binding β-subunits in F_1 is indicated. If yeast F_1 contains three β-subunits (Ref. 23 and our own labelling data, still to be published), this means that two β-subunits differ in some way from the third, even after dissociation with LiCl. One possibility is that this is due to a pre-existing or ligand-induced conformation difference associated with the presence of high-affinity sites for adenine nucleotides [30]. Indeed we have found recently that tightly bound nucleotides are still present after treatment with LiCl (Berden, J.A. and Mioch, L., unpublished). Possible explanations for the paradox that the isolated β-subunit binds aurovertin stoicheiometrically are (1) the third β-subunit sticks to the column during the isolation procedure (our yield of protein was only about 60%) or (2) the conformation of this subunit changes to that of the others, e.g. by dissociation of a bound adenine nucleotide or one of the other subunits of F_1. The β-subunit isolated from yeast is much less stable than that from heart, and, in our hands, the presence of ATP both during and after the isolation is essential for retention of the aurovertin binding. The modification of the β-subunit in the absence of ATP is not reversed by subsequent addition of ATP. The instability of the β-subunit is probably the reason why Douglas et al. [18] found only 0.38 mol binding sites per mol β-subunit. A clear difference with the results of Douglas is that we find a much smaller enhancement of the aurovertin fluorescence on binding to the isolated β-subunit than on binding to intact F_1. Our results are in agreement with the results on beef heart F_1 [13,14].

Although we were not able to confirm the conclusion of Ryrie and Gallagher [31] that the polypeptides with molecular weight around 40 000 are breakdown products of the α- and β-subunits of the enzyme, we can confirm their evidence that these polypeptides are impurities, since they can be largely

removed using gel chromatography, with a concomitant increase in specific activity. (However, see Ref. 23).

The effects of methanol on the binding of aurovertin can be described on the basis of a model in which both F_1 and aurovertin are considered to be distributed over aqueous and methanol phases. So far as the mathematical treatment is concerned, this is identical with a model in which methanol binds to F_1. An effect of methanol on the measured K_d of the binding of aurovertin to F_1 may be expected, since aurovertin is more soluble in methanol than in water, and this will result in an apparently lower affinity for F_1. From the loss of aurovertin-binding sites in the presence of methanol, we conclude that aurovertin does not bind to sites occupied by methanol (or in a methanol phase). A direct competition between methanol and aurovertin can be excluded, since in that case methanol would not decrease the concentration of sites. Apparently methanol alters the conformation of the region of the binding site, so that it no longer binds aurovertin. The different conformation of this region, however, has no influence on the catalytic region, since the maximum velocity is not influenced by methanol. The finding that, in the presence of sulphite, methanol has no effect on the ATPase activity is in agreement with the idea that the effect of methanol on the activity is indeed due to its effect on the regulatory site. Since sulphite has no effect on the binding of aurovertin, whether ATP is present or not, it may be concluded that changes at the regulatory site do not influence the aurovertin-binding site. The effect of ATP (lowering the quantum yield of the aurovertin fluorescence) must, then, be due to binding to the catalytic site. That methanol affects both the aurovertin-bindng and the regulatory sites, must be ascribed to its being less specific.

The slowly developing inhibition of the ATPase activity in the presence of aurovertin makes it clear that a complex enzyme like F_1 can undergo multiple conformational changes. On the addition of aurovertin at least three different conformations of the F_1-aurovertin complex can be distinguished: the very rapidly formed complex, a secondary complex with increased fluorescence (not formed with the isolated β-subunit), and a tertiary inhibited complex that is formed only under turnover conditions. This implies that under turnover conditions the conformation of the aurovertin-binding site differs from that in resting state.

The large effect of methanol on the inhibition by aurovertin of the ATPase acitvity suggests that under conditions of turnover the conformation of the aurovertin binding site is much more sensitive to methanol than in the resting state (6-times more sensitive, Fig. 8) or that the equilibrium between the inhibited and non-inhibited form of the aurovertin complex is shifted by methanol towards the non-inhibited form, without, however, affecting the $t_{1/2}$ for the formation of the inhibitory complex.

Since Penefsky [32] has reported that the inhibition of ATPase or ITPase activity by MgADP can be released by 15% methanol, it is possible that the binding site responsible for this inhibition and the aurovertin-binding site are identical.

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